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Short communication

A short motif in the N-terminal part of the coat protein is a host-specific determinant of systemic infectivity for two potyviruses

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SUMMARY

Although the biological variability of *Watermelon mosaic virus* is limited, isolates from the three main molecular groups differ in their ability to infect systemically *Chenopodium quinoa*. Mutations were introduced in a motif of three or five amino acids located in the N-terminal part of the coat protein, and differing in isolates from group 1 (motif: lysine-glutamic acid-alanine (Lys-Glu-Ala) or KEA, systemic on *C. quinoa*), group 2 (Lys-Glu-Thr or KET, not systemic on *C. quinoa*) and group 3 (KEKET, not systemic on *C. quinoa*). Mutagenesis of KEKET in an isolate from group 3 to KEA or KEKEA was sufficient to make the virus systemic on *C. quinoa*, whereas mutagenesis to KET had no effect. Introduction of a KEA motif in *Zucchini yellow mosaic virus* coat protein also resulted in systemic infection on *C. quinoa*. These mutations had no obvious effect on the disorder profile or potential post-translational modifications of the coat protein as determined *in silico*.

The genus *Potyvirus*, containing single positive-stranded monopartite RNA viruses, represents about 20% of all known plant viruses. The 10-kb genome encodes a large polyprotein that is cleaved autocatalytically in 10 functional polypeptides, and an extra protein (PIPO) is expressed by an internal frameshift (Chung *et al*., 2008). Taken together, potyviruses infect most dicot and monocot crops, but each virus species usually has a limited host range. The host range of each virus, i.e. its ability to replicate in a cell, move first to neighbouring cells and then enter the phloem and infect systemically different hosts, is determined by interactions between host and virus components that are only partly known. Pathogenicity determinants are spread along the genome of potyviruses (Carbonell *et al*., 2013). Among these determinants, at least five viral proteins have been shown to play a role in systemic movement: helper component-proteinase (HC-Pro) (Cronin *et al*., 1995), PIPO (Wen and Hajimorad, 2010), 6K2, viral genome-linked protein (VPg) (Rajamäki and Valkonen, 1999) and coat protein (CP) (Dolja *et al*., 1995), occasionally in a host-specific manner (Andersen and Johansen, 1998; Carbonell *et al*., 2013; Saenz *et al*., 2002; Spetz and Valkonen, 2004). More precise characterization of the host-specific determinants of systemic movement is important to understand viral gene functions and virus–host interactions. In this study, we characterized a short motif in the N-terminal part of the CP of *Watermelon mosaic virus* (WMV) that modulates the systemic infection of *Chenopodium quinoa* without affecting its infectivity on other hosts, and showed that the same motif could also drive systemic infection of *C. quinoa* by *Zucchini yellow mosaic virus* (ZYMV).

WMV and ZYMV are among the most common and damaging cucurbit potyviruses worldwide (Lecoq and Desbiez, 2008). WMV presents a high molecular variability, with three distinct groups of isolates—named groups G1–G3—presenting about 10% intergroup nucleotide difference (Desbiez *et al*., 2007), and a large number of intergroup recombinants (Desbiez and Lecoq, 2008; Desbiez *et al*., 2011). G1 isolates usually have a 'KEA' motif at the N-terminal extremity of their CP (Nter CP), four residues before the 'DAG' motif involved in aphid transmission (Atreya *et al*., 1991), whereas G2 and G3 isolates have a 'KET' and 'KEKET' motif, respectively (Desbiez *et al*., 2007, 2009). Despite its important molecular diversity, the biological variability of WMV is limited: in a study including 48 different crop and weed species, only two hosts, *Ranunculus sardous* and *C. quinoa*, revealed differences between the three groups (Lecoq *et al*., 2011). G1 isolates induced systemic chlorotic spots on *C. quinoa*, whereas G2 and G3 induced local lesions, but failed to infect *C. quinoa* systemically. G3 isolates induced latent systemic infection in *R. sardous*, whereas G1 and G2 isolates failed to do so. Two natural G1/G3 recombinants of WMV, C05-463 and C07-349, were found in France in 2005 and 2007, respectively. Both had a recombination point within or close to the KEKET/KEA motif in the CP, but the recombination yielded a 'KEA' motif in C07-349 and a 'KET' motif in C05-463 (Desbiez *et al*., 2009, 2011). Interestingly, the recombinant displaying the 'KEA' motif was systemic on *C. quinoa*, whereas the 'KET' recombinant was not, both being unable to infect *R. sardous* systemically (Desbiez *et al*., 2011). This suggests that the 'KEA' motif could be involved in systemic infection of *C. quinoa*.

In the case of ZYMV, most isolates induce only local chlorotic lesions in *C. quinoa*, but isolate R5A from Réunion Island infects

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this host systemically (H. Lecoq, unpublished data). Although most isolates do not have any 'KE' motif upstream from 'DAG', isolate R5A displays a 'KEA' motif just before 'DAG' (Fig. S1, see Supporting Information). In order to validate the function of the KEA motif in WMV and ZYMV systemic infection on *C. quinoa*, this motif was introduced by site-directed mutagenesis in an infectious clone of WMV strain FMF00-LL1 containing two introns in the P3 and CI coding regions (Desbiez *et al*., 2012), as well as in an intronless clone of ZYMV strain E15 (Desbiez *et al*., 2012).

For WMV, a 1.5-kb fragment of FMF00-LL1 overlapping Nter CP was subcloned into pBlueScript(KS+) using *Eco*RI and *Xho*I restriction sites. Mutagenesis was performed by polymerase chain reaction (PCR) on the 4.5-kb construct using three sets of primers (Table S1, see Supporting Information), in order to obtain 'KEA', 'KET' and 'KEKEA' motifs, respectively. The PCRs were performed with *Pfu* DNA polymerase (Promega, Madison, WI, USA). A denaturation of 3 min at 92 °C was followed by 30 cycles of 45 s at 92 °C, 1 min at 55 °C and 12 min at 68 °C. The PCR products were digested with *Dpn*I in order to eliminate the nonmutated matrix, and then used for the transformation of *Escherichia coli* DH5α. The presence of the expected mutations in the plasmids was checked by partial sequencing. The 1.5-kb inserts were released by digestion with *Xho*I + *Eco*RI, and introduced by homologous recombination in yeast (Desbiez *et al*., 2012) in the complete FMF00-LL1 infectious clone digested with *BamH*I and *Nae*I.

For ZYMV, the mutagenesis was performed by fusion PCR. Two PCRs were performed on the ZYMV-E15 infectious clone using Pfu DNA polymerase: (i) ZYMV-7500-5' + ZYMV-KEA-3'; and (ii) ZYMV-KEA-5' + ZYMV-CP-3', where ZYMV-KEA-5' and ZYMV-KEA-3' are mutagenic primers (Table S1). A denaturation at 95 °C for 5 min was followed by 35 cycles of 45 s at 95 °C, 45 s at 50 °C and 3 min at 72 °C, and a final elongation of 7 min at 72 °C. PCR fragments of the expected sizes (1100 and 232 bp, respectively) were excised from agarose gel, eluted in 10 μL of Tris (10 mM, pH 8.0), and 0.25 μL of each fragment was used for fusion PCR with external primers ZYMV-7500-5' and ZYMV-CP-3', employing the same cycle conditions as above. The 1294-bp mutagenized fragment was introduced by homologous recombination in yeast into the ZYMV-E15 infectious clone digested with *Sac*I and *Mlu*I.

After extraction of yeast DNA and transformation of *E. coli*, the WMV and ZYMV constructs were employed for the inoculation of zucchini squash cv. 'Diamant' using a 'GeneGun' (Gal-On *et al*., 1995). Total RNA was extracted from 0.1 g of symptomatic plants 2 weeks after inoculation using TRI-reagent (Molecular Research Center, Cincinnati, OH, USA). RT-PCR was performed with primers WMV-5' + WMV-3' and ZYMV-CP-5' + ZYMV-CP-3' overlapping the Nter CP for WMV and ZYMV, respectively, according to standard protocols (Lecoq and Desbiez, 2012); the presence of the expected mutations was checked by partial sequencing.

Three weeks after bombardment, 1 g of infected zucchini leaf was ground in 4 mL of cold Na_2HPO_4 (0.03 M) +

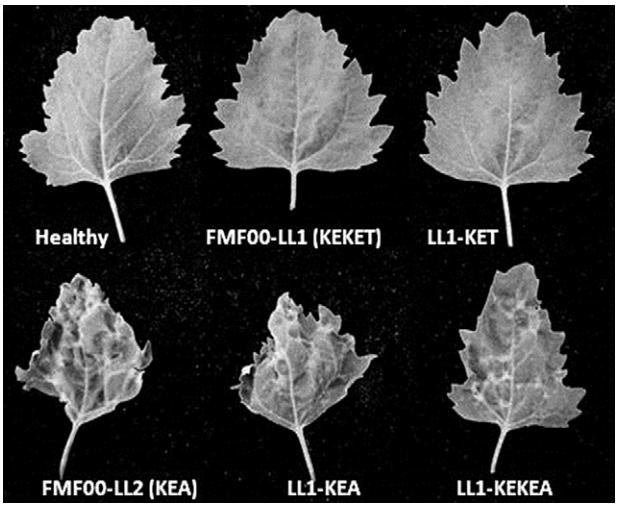

Fig. 1 Symptoms of the different *Watermelon mosaic virus* (WMV) constructs on *Chenopodium quinoa* upper leaves, 4 weeks after mechanical inoculation. The leaves were taken at the same level for all conditions.

diethyldithiocarbamate (0.2%). Activated charcoal and carborundum (75 mg/mL each) were added before mechanical inoculation of two 4-week-old *C. quinoa* plantlets, as well as two melon (cv. 'Védrantais') and two zucchini squash (cv. 'Diamant') plantlets at the cotyledonary stage as controls. WMV isolates FMF00-LL1 (not expected to be systemic on *C. quinoa*) and FMF00-LL2 (systemic), as well as ZYMV isolates E15 (not systemic) and R5A (systemic), were inoculated as negative and positive controls, respectively. Symptoms were checked visually, and the presence of the viruses in inoculated or systemic leaves was estimated by double antibody sandwich-enzyme-linked immunosorbent analysis (DAS-ELISA) with specific WMV and ZYMV antisera, according to Lecoq *et al*. (2011). The experiments were repeated three times.

All WMV and ZYMV constructs were infectious in zucchini squash after GeneGun inoculation. After mechanical inoculation, symptoms of the WMV mutants were indistinguishable from those of the parental isolate FMF00-LL1 on zucchini and melon, and the kinetics of symptom appearance were similar to those of FMF00-LL1 (data not shown). In *C. quinoa*, all WMV strains and mutants induced chlorotic spots in the inoculated leaves. In apical leaves, the mutants LL1-KEA and LL1-KEKEA displayed, in all the inoculated plants, chlorotic spots and leaf deformation indistinguishable from those induced by the natural 'KEA' strain FMF00-LL2 (Fig. 1), whereas, for FMF00-LL1 ('KEKET' motif) and the LL1-KET mutant, the apical leaves showed no symptoms and the virus was not detected by ELISA at the apex of the plants. Partial sequencing of virus isolates from the apex of *C. quinoa* plants inoculated with LL1-KEA and LL1-KEKEA confirmed the presence of the 'KEA' and 'KEKEA' mutations, respectively (data not shown). For ZYMV, both the R5A isolate and the E15-KEA mutant induced systemic chlorotic spots in *C. quinoa,* whereas E15 did not induce any systemic symptoms and was not detected by ELISA at the apex of

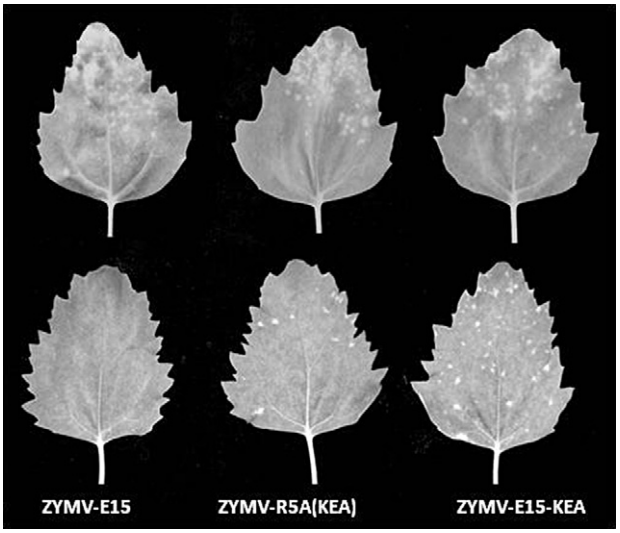

Fig. 2 Symptoms of the different *Zucchini yellow mosaic virus* (ZYMV) strains and constructs on *Chenopodium quinoa*, 2 weeks after mechanical inoculation. Top: inoculated leaves; bottom: upper leaves from the same plants.

the plants (Fig. 2). The presence of the 'KEA' motif in the apical leaves of *C. quinoa* plants inoculated with the E15-KEA mutant was confirmed by partial sequencing (data not shown). All *C. quinoa* plants inoculated with WMV or ZYMV presented numerous local lesions (not quantified) on the inoculated leaves, indicating that the lack of systemic infection with some constructs was not caused by a mis-inoculation.

In order to estimate the natural diversity and selective constraints on WMV and ZYMV CP, the nonidentical complete CP sequences of 93 and 135 isolates of WMV and ZYMV, respectively, available in GenBank, were aligned using CLUSTALW included in DAMBE (Fig. S1), and variability in the translated amino-acid sequences was checked visually. Variability in the Nter CP was also studied on partial sequences from 1839 WMV isolates and 231 ZYMV isolates characterized during molecular epidemiology surveys in France between 2004 and 2008 (Desbiez *et al*., 2009; Joannon *et al*., 2010; Lecoq *et al*., 2009). Among the 93 WMV CP sequences of natural isolates used in the analyses, 54 (58.1%) had the 'KEA' motif, whereas 19 (20.4%) had a 'KET' and 20 (21.5%) a 'KEKET' motif (Fig. S1). For the 1839 partial CP sequences, the KEA, KET and KEKET motifs were present in 707, 58 and 1069 isolates, respectively (frequencies of 38.4%, 3.2% and 58.1%, respectively). The five remaining isolates (0.3% of the samples) displayed a TEKET (four isolates from the same field) or KERET (one isolate) motif. No natural isolate with a 'KEKEA' motif was detected, suggesting that this mutation has a fitness cost, although this was not obvious in single infections in our glasshouse conditions. Thus, more than 99.7% of all WMV isolates sequenced so far had one of the three motifs 'KEA', 'KET' or 'KEKET'. The high frequency of the 'KEKET' motif in France is related to the emergence of G3 isolates that usually have this motif, whereas the frequency of G1 ('KEA' motif) has decreased, and G2 isolates (usually presenting the 'KET' or sometimes 'KEA' motif) were rare in the 2004–2008 survey (Desbiez *et al*., 2009).

Most of the 135 ZYMV CP sequences did not have a 'KE' motif upstream from 'DAG' in the Nter CP (Fig. S1), even though, as in most potyviruses, repeats of charged residues are present downstream from DAG (data not shown). Only a few isolates from Réunion Island, Australia and Vietnam had a 'KEA' or 'REA' motif. With the exception of the R5A isolate from Réunion Island, their infectivity on *C. quinoa* is not known. Among the 231 partial CP sequences from epidemiological surveys in France, no isolate with a 'KEA' motif was observed.

In WMV, the introduction of a 'KEA' mutation did not have any impact on virus symptoms or accumulation in cucurbits, as determined by DAS-ELISA (data not shown), whereas, in ZYMV, the 'KEA' mutants presented delayed symptom appearance on zucchini squash and milder symptoms. This is consistent with the fact that 'KEA' isolates of WMV are very common in natural conditions—they represent the large majority of G1 isolates—and do not present obvious fitness defects compared with other isolates (Lecoq *et al*., 2011), even if G1 isolates are currently being replaced by G3 in southern France (Desbiez *et al*., 2009; Fabre *et al*., 2010). On the contrary, very few ZYMV isolates have a 'KEA' motif. Isolate R5A is highly divergent molecularly from the common 'group A' isolates of ZYMV present worldwide, and has several mutations in the Nter CP besides KEA (Fig. S1). This may prevent the fitness defects observed for the laboratory-made E15-KEA mutant.

For selection studies on the 93 complete WMV CP sequences, positions 13–18, corresponding to the second 'KE' repeat in WMV sequences from group G3 (invariable AAAGAA in all sequences available), were deleted in order to avoid gaps in the alignment. In addition, isolate TW-NT1 (AF127933) was removed from the ZYMV alignment because it has a two-amino-acid deletion in the Nter CP. The best substitution model was determined for each virus with the model selection tool of the DataMonkey web interface (http://www.datamonkey.org) of the HyPhy package (Kosakovsky Pond *et al*., 2005). Search for residues under positive or negative selection was performed using the SLAC, FEL and IFEL software packages available with Datamonkey. These methods estimate, for each codon, the synonymous (dS) and nonsynonymous (dN) substitution rates. If $dN < (\text{or} >) dS$, a codon is considered to be negatively (or positively) selected. A *P* value derived from a two-tailed extended binomial distribution is used to assess significance.

No codon under positive selection was detected in the Nter CP of ZYMV or WMV. The average dN/dS values in the CP were 0.105 and 0.117 for WMV and ZYMV, respectively, suggesting that most codons are under negative selection, as expected for a structural protein. dN/dS values were 0.303 (WMV) and 0.392 (ZYMV) for the variable Nter CP alone, confirming that it is less constrained than the rest of the protein (Shukla and Ward, 1989).

Five methods were used to predict the intrinsic disorder of WMV and ZYMV CP and complete genomes: (i) FoldIndex (http://bip.weizmann.ac.il/fldbin/findex), which computes the charge/hydropathy ratio along proteins; (ii) Disopred (http://bioinf.cs.ucl.ac.uk/disopred); (iii) DisEMBL (http://dis.embl.de); (iv) GlobProt (http://globplot.embl.de); and (v) PONDR® (http://www.pondr.com), as described by Hébrard *et al*. (2009). Access to PONDR® was provided by Molecular Kinetics (Indianapolis, IN, USA)

Similar results were obtained with the five methods. Three regions of the WMV and ZYMV genomes appeared to be highly disordered: the P1 protein, VPg, as already known for potyviruses (Grzela *et al*., 2008; Hébrard *et al*., 2009; Rantalainen *et al*., 2008), and the Nter CP (data not shown). The 'core' part of the CP appears to be structured, as already estimated for potyviruses (Kendall *et al*., 2008). Intrinsic disorder in proteins or protein regions can confer a functional diversity and an adaptive advantage by enabling interaction with many different molecular partners (Chen *et al*., 2008; Rantalainen *et al*., 2011). For WMV, the 'KEKET' to 'KEA' mutation modified slightly the disorder profile of the polypeptide (data not shown). However, when comparing the disorder profiles of the 'KEKET', 'KEKEA', 'KET' and 'KEA' variants, the differences in disorder profiles appeared to be related to the 'KE' insertion/deletion between the motifs, rather than to the A to T substitution (data not shown). For ZYMV, the disorder profiles of ZYMV-E15, ZYMV-E15-KEA and ZYMV-R5A appeared to be very similar despite sequence differences.

In addition to structure or charge differences, not obvious here, differential post-translational modifications could play a role in the biological effect of the 'KEA' motif in WMV and ZYMV. It has been shown that serine (Ser), threonine (Thr) and tyrosine (Tyr) residues in the CP of potyviruses can be phosphorylated (Ivanov *et al*., 2001) or modified by *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) (Fernandez-Fernandez *et al*., 2002), and such post-translational modifications can affect viral infectivity (Ivanov *et al*., 2003; Kim *et al*., 2011). Interestingly, *O*-GlcNAc glycosylated of T residues in the N-terminal part of *Plum pox virus* (PPV) CP has been shown recently to enhance virus infection in a host-specific manner (Pérez *et al*., 2013). The presence of such modification sites in or around the 'KEA' motif was tested *in silico*. Phosphorylation at Ser, Thr or Tyr residues was estimated with NetPhos2 (Blom *et al*., 1999) and with DEPP (Disorder Enhanced Phosphorylation Prediction) included in PONDR®. Sites predicted to be *O*-GlcNAc glycosylated as well as phosphorylated, and which may be reversibly and dynamically modified by *O*-GlcNAc or phosphate groups at different times in the cell, were predicted with YinOYang 1.2 (Gupta and Brunak, 2002).

There was no evidence for phosphorylation or *O*-GlcNAc modification of the 'T' in the 'KEKET' or 'KET' motif in WMV using NetPhos2 and YinOYang. However, a putative phosphorylation of this residue was predicted with DEPP (DEPP score = 0.65). Although this observation needs to be validated by biochemical and biological approaches, it may contribute to explain the differential infectivity of WMV isolates on *C. quinoa*. For ZYMV, the 'T' in the 'TVA' motif replaced by the 'KEA' in R5A was not a candidate for phosphorylation. However, it was predicted to be potentially *O*-GlcNAc-modified with YinOYang 1.2. The 'TV' to 'KE' mutation did not modify the phosphorylation/*O*-GlcNAc glycosylation profile of neighbouring residues (data not shown).

In this work, we have shown that a 'KEA' motif in the Nter CP of two different *Potyvirus* species, WMV and ZYMV, is involved in their ability to infect systemically *C. quinoa*. The Nter CP, one of the most variable regions in the *Potyvirus* genome, is involved in virus systemic movement (Dolja *et al*., 1995). However its net charge appears to be more important than its primary sequence for this function (Kimalov *et al*., 2004; Lopez-Moya and Pirone, 1998). Replacing the N-terminal part of ZYMV CP by that of WMV (G2 isolate), or that of PPV with that of ZYMV, yielded infectious chimeric viruses with biological properties similar to those of the parental isolates (Tobias *et al*., 2001; Ullah *et al*., 2003). The N-terminal part of ZYMV CP could even be partially deleted or replaced by a nonviral peptide without losing systemic infectivity in cucurbits (Arazi *et al*., 2001). In contrast, a point mutation in the Nter CP determined the ability of *Pea seed-borne mosaic virus* to infect *C. quinoa* systemically (Andersen and Johansen, 1998). Both point mutations and deletions in PPV Nter CP also conferred the ability to infect systemically *Nicotiana benthamiana* and *N. clevelandii*, with or without fitness loss on *Prunus* hosts (Carbonell *et al*., 2013). The Nter CP was also a determinant of *Potyvirus* ability to overcome the RTM resistance in *Arabidopsis thaliana* characterized by a restriction of long-distance movement (Decroocq *et al*., 2009). Several amino acids in Nter CP of the *Tritimovirus Wheat streak mosaic virus* were also shown to affect the long-distance movement of the virus in a host- and strain-specific manner (Tatineni *et al*., 2011). This confirms the importance of the Nter CP in host-specific long-distance movement, not only in potyviruses, but also in other members of the *Potyviridae* family. In the aforementioned situations, the amino acid(s) important for virus movement were more internal than in the case of WMV and ZYMV, and located downstream from the 'DAG' triplet. More studies are needed to estimate whether the KEA mutation contributes to modified CP properties and differential interactions with specific plant components involved in virus long-distance movement.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Sequence variability around the 'KEA' motif in the N-terminal part of the coat protein (CP) of *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV).

Table S1 Primers used to introduce mutations in *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV) infectious clones.